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ASSEMBLED CHEMICAL WEAPONS ASSESSMENT (ACWA) PROGRAM, IMMOBILIZED CELL BIOREACTOR TOXICITY MONITORING

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Until recently, incineration was the only proven technology, adopted by the U.S. Army, that successfully destroyed chemical agents. However, public opposition to incineration redirected research to evaluate alternative technologies. Through the Alternative Technology Program, the U.S. Army adopted biodegradation as a proven method for destroying the mustard stockpile at Aberdeen Proving Ground. However, research did not address the destruction of mustard when mixed with explosives generated from the destruction of Assembled Chemical Weapons. The Assembled Chemical Weapons Assessment (ACWA) Program was tasked to find alternatives to the incineration/destruction of assembled chemical weapons. The ACWA Program has been evaluating biodegradation to determine if tetrytol (explosive) will disrupt the biodegradation process. Microtox (MTX) assays were used to monitor changes in toxicity due to changes in feed composition. The MTX assay uses a luminescent marine bacteria (Vibrio fischeri) that can survive high salt concentrations. When subjected to toxic substances, cellular respiration decreases, resulting in a corresponding decrease in luminescence. Feed and effluent samples were monitored for toxicity using the MTX assays over a 42-day period.

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PREFACE

The work described in this report was authorized under Sales Order No. 9E9994, Assembled Chemical Weapons Assessment (ACWA) Program. This work was started in July 2000 and completed in September 2000.

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CONTENTS

FIGURES

TABLES

ASSEMBLED CHEMICAL WEAPONS ASSESSMENT (ACWA) PROGRAM, IMMOBILIZED CELL BIOREACTOR TOXICITY MONITORING

1. ### INTRODUCTION

In 1996, congress established the Assembled Chemical Weapons Assessment (ACWA) program to address public concerns about the destruction of the chemical weapons stockpile. The program was tasked to identify an alternative method to incineration for disposing of U.S. Army munitions filled with chemical agents. One alternative identified by the ACWA as having potential is neutralization followed by biodegradation. This process was first demonstrated by Harvey et. al.,[1] to successfully biodegrade hydrolyzed mustard in Sequencing Batch Reactors. Further research by Guelta and DeFrank[2] demonstrates the success of immobilizing biomass on a fixed surface to degrade hydrolyzed mustard. The U.S. Army has since adopted this process and is currently implementing the technology to destroy the HD chemical stockpile at U. S. Army Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground (APG), MD. Harvey's research did not address the impact on the biodegradation process when mixed with energetics.

Water Hydrolysis of Energetic and Agent Technology (WHEAT) is an alternative to incineration proposed for destroying chemical warfare munitions (M60 and 105 mm projectiles) containing mustard and energetics.[*] WHEAT technology has been demonstrated to successfully maintain a biodegradation process using hydrolyzed mustard mixed with hydrolyzed energetics. The process uses a 1000 gal Immobilized Cell Bioreactor (ICB) containing three chambers (developed by Parson's/Honeywell). Feed is directed to chamber 1, reaches a critical volume and flows to chamber 2, then to chamber 3, and flows out as effluent. Chamber 1 is the most biologically robust chamber since it is the first to be subjected to potentially toxic additions. If an unknown toxicant reduces the efficiency of the first chamber, it is assumed that chambers 2 and 3 will complete the biodegradation process, therefore maintaining a steady state. A schematic of the process can be seen in Figure 1. The feed components were added to the feed mixing tank and pumped into the ICB at a constant rate over a 24-hr period. The Hydraulic Resonance Time (HRT) feed entered the ICB and emerged as an effluent in approximately 5 days. The effluent was then processed into brine and recycled water. For a more detailed description of the ICB and its' operations, see Guelta et. al.[3]

The degradation process was monitored through the analysis of chemical oxygen demand (COD), total organic carbon (TOC), organic compounds, and various water parameters. However, the study was not designed to monitor toxicity simultaneously. Monitoring effluent toxicity may provide an additional check on the bioprocess and provide information that can be used in determining other possible waste management options. In past studies, toxicity studies were conducted by Haley et. al.[4] using effluents produced from Sequencing Batch Reactors (SBRs). The SBRs were fed hydrolyzed HD obtained from the ton container stockpile at

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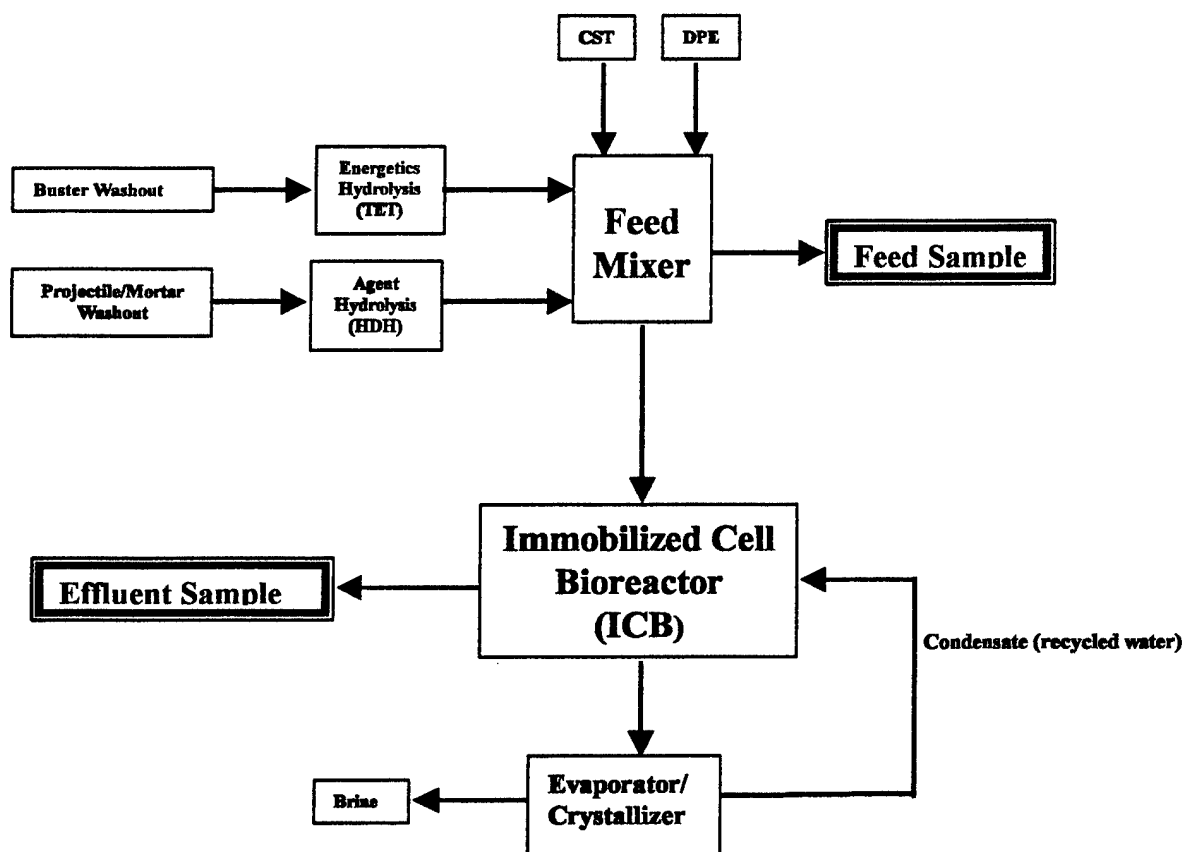
[*] PM, ACWA, ATTN: AMSSB-PM-ACWA, APG, MD.

Figure 1. Schematic of the ICB Showing Sampling Points for Toxicity Monitoring.

APG, MD. The results of that study provided regulators the option to dispose of bioeffluent through a Waste Water Treatment Facility (WWTF). The information obtained from monitoring the toxicity of ICB effluents may ultimately be used in determining other possible waste management options as well.

The ACWA program has employed WHEAT as an alternative for destroying chemical weapons. The study was not designed to evaluate the ability of Microtox (MTX) to monitor effluent toxicity. The work described in this report was a side-line project used to gain information on the feasibility of using the MTX assay to monitor toxicity.

In this study, the MTX assay was used to monitor toxicity of feed and effluent to determine if toxicity monitoring to assess the performance of the ICB is feasible. The MTX assays are quick/inexpensive tests that have proven to be very sensitive to hydrolyzed HD and routinely capable of discriminating between untreated, partially biotreated, and fully biotreated hydrolysate.[5] The MTX bioassay exposes a bioluminescent marine bacterium (*Vibrio fischeri*) to a sample of unknown toxicity and measures the change in light output as the means of determining effects on the organism. A reduction in light output is a direct indication of metabolic inhibition.

2.   METHODS AND MATERIALS

The MTX bacterium *Vibrio fischeri* was cultured by Azur Environmental (Carlsbad, CA), shipped in lyophilized form, and stored frozen. The MTX bacterium was rehydrated immediately before testing and used up to 2 hr after rehydration. Each bioassay used < 3 mL of sample and was performed in a temperature controlled photometer. Due to interference caused by suspended particulate/biomass, the samples were centrifuged for 10 min at 500 relative centrifugal force (RCF). Then, the supernatant was decanted and used in testing. The samples were diluted with MTX diluent, and pH adjustments were done using 10% HCl as needed. The assays were performed in glass cuvettes in temperature-controlled wells of a photometer. The assay had to have a minimum of four dilutions exhibiting a dose response for optimum accuracy in predicting toxicity. The addition of bacteria was referred to as time zero. At 5 and 15 min, the control and treatment groups were analyzed for light output. Data was analyzed using the MTX Test Protocol software to determine the EC_{50} (the effective concentration causing a 50% reduction in light output).

Until needed, the feed components (Table 1) were stored separately at room temperature in 55-gal drums. Hydrolyzed mustard (HDH) and hydrolyzed tetrytol (TET) feed components were representative of the M60 and 105 mm projectile drained munitions. The CST and DPE were products of the high pressure steam process used in cleaning the metal parts remaining from the munitions. The feed component ratios and the day the feed formulation was changed are listed in Table 2.

Table 1. Components Used in the Various Feed Configuration

HDH   Hydrolyzed Mustard (predominantly thiodiglycol)

TET   Tetrytol (2,4,6-trinitrophenyl-N-methylnitramine + Trinitrotoluene)

CST   Continuous Stream Treatment with shredded wood pallets

DPE   Continuous Stream Treatment with shredded wood pallets and designated protective equipment (PVC and rubber suits)

Table 2. Summary of Feed Composition. The feed composition changed on the day listed in the table below.

Day	HDH (gal)	TET (gal)	CST (gal)	DPE (gal)	H2O (gal)
100	40	1.8	0.5	0.0	134.5
112	40	1.8	2.0	0.0	133.0
114	40	1.0	2.0	0.0	132.0
119	45	1.0	0.0	2.0	127.0
126	50	1.0	0.0	2.0	122.0
127	50	1.0	3.0	0.0	121.0
130	50	1.0	2.0	1.0	121.0
131	50	1.0	0.0	3.0	121.0
141	50	1.0	0.0	2.0	122.0
142	50	1.0	0.0	0.0	124.0
144	50	0.75	0.0	0.0	125.0

3.   RESULTS

The feed formulation changed 11 times (Table 2) during the period when MTX sampling was conducted (42 days). In Figure 2, the EC_{50} values of the various feed formulations have been grouped and plotted. The average EC_{50} for all the feed samples was $0.8 \pm 0.3\%$ vol/vol with a range of 1.6%. The feed was approximately one order of magnitude more toxic than methanol ($EC_{50} = 5.6\%$) and acetone ($EC_{50} = 2.3\%$). One sample each in feed formulation groups 1 and 10 was determined to be an outlier using the mean $\pm 2 \times SD$ method. However, after reviewing the experiment conditions and possible computation errors, there was no substantial reasoning to eliminate these data points. Therefore, the data points were included in the statistical calculations. Due to the limited sample size in several formulation groups, the normality test failed, and the Kruskal-Wallis ANOVA was conducted to determine if there were differences between feed formulations. The results showed no differences between these formulations. The components of the feed were subjected to toxicity tests to determine if one or more of the feed ingredients were the major causes of toxicity. In Figure 3, the toxicity of the feed components was plotted along with several other process streams. The bars for each component in Figure 3 represent the mean from each group. However, since the effluent was constantly changing due to change in feed formulations, the effluent bar represents samples taken towards the end of the study. The CST was the most toxic component having an $EC_{50} = 0.4\%$ with tetrytol ($EC_{50} = 1.0\%$), hydrolyzed HD ($EC_{50} = 1.9\%$), and DPE ($EC_{50} = 2.7\%$), in that order, being the next toxic. However, statistical results showed CST and tetrytol as not being significantly different at $p \leq 0.05$, while DPE was different than CST and tetrytol. Therefore, CST and tetrytol were similar in toxicity and the most toxic components of the feed. Hydrolyzed HD and DPE were not significantly different and were the least toxic feed components.

Preliminary effluent sampling was started on day 106 and continued intermittently for 41 days. The effluent toxicity ranged from 3.1% on day 106 to 21.7% on day 147. In Figure 4, the toxicity of effluent samples was plotted along with the feed toxicity to show the magnitude of toxicity reduction over time. The ICB reduced the toxicity of the feed throughout the entire study. The toxicity decreased over time; however, several negative peaks on days 115, 123, 127, and 136 were seen. Attempts were made to correlate the negative peaks to equipment failure. After reviewing the ICB maintenance log books, there were no mechanical failures that could be matched to the negative peaks. The peaks on days 115 and 127 have a one point dip and may be an artifact. However, the negative peaks on days 119 and 123 have several points forming a negative trend. These appear to be real increases (negative trend) in toxicity.

Monitoring COD and conducting chemical analysis of the effluent is how ACWA evaluated the status and performance of the ICB. Since COD was less costly and required little time, it was monitored daily. During this project, if the reduction in COD was 90% or greater, the ICB was considered to be working properly. During the 42 days of toxicity sampling, the feed formulation was changed seven times, and the ICB was able to maintain COD reduction over 90%.
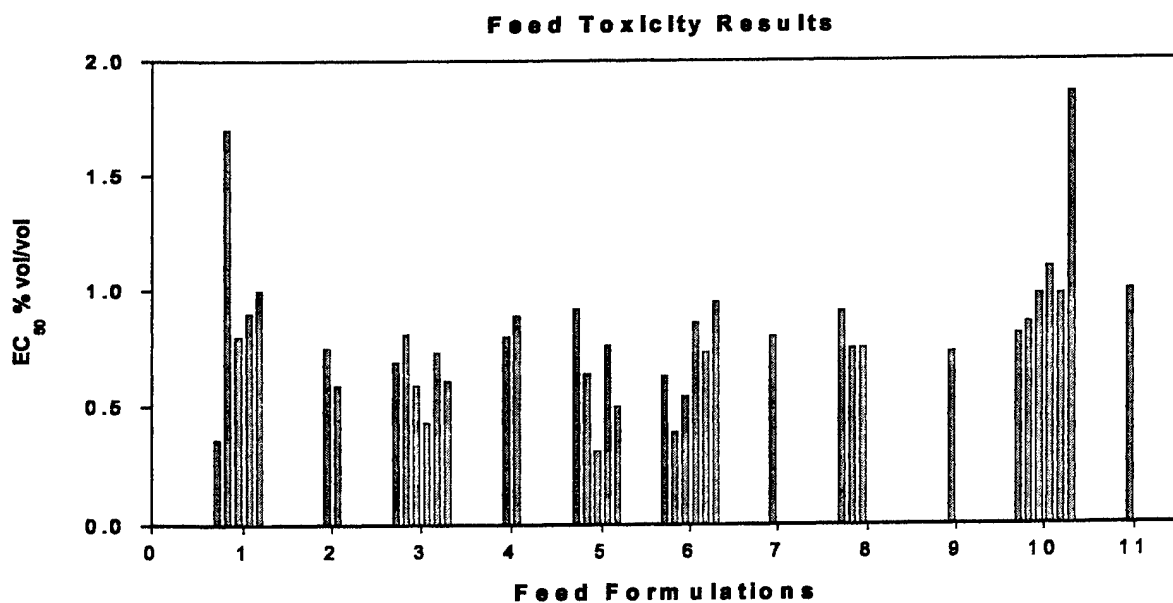
Feed Toxicity Results

Figure 2. Samples Taken from the Various Feed Formulations and Tested Using MTX Bioassays. The outliers in groups 1 and 10 were included in the statistical analysis.

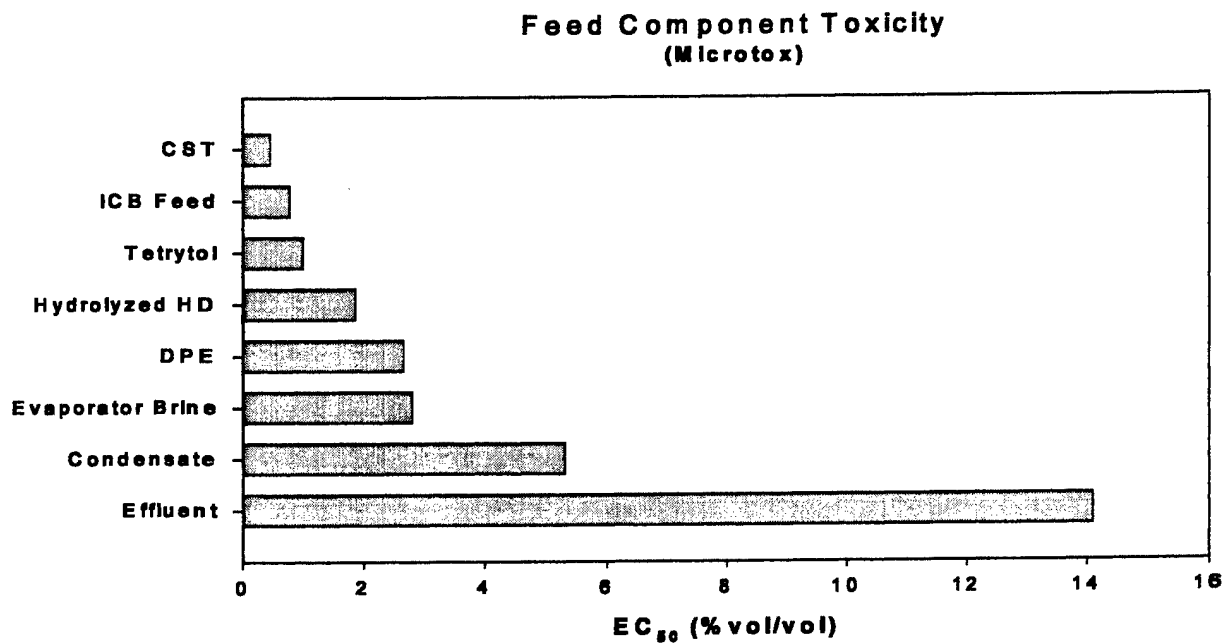**Feed Component Toxicity**
(Microtox)

Figure 3. Toxicity of Various Feed Components and Several Waste Streams (Condensate and Evaporator Brine) Produced by the Biodegradation Process.
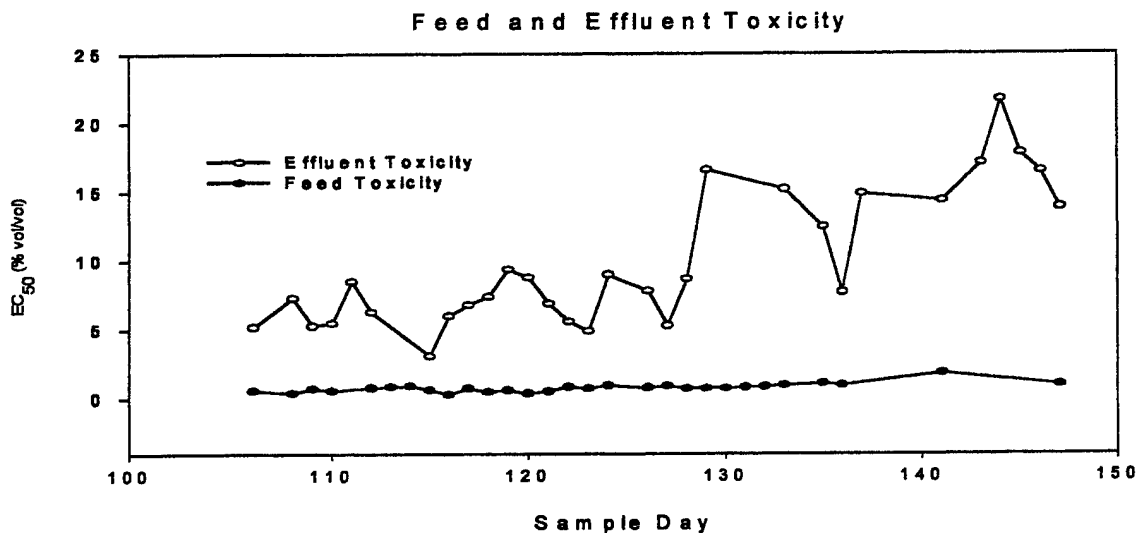
Figure 4. Feed and Effluent Toxicity Representing Days 105 Through 147.

If the change in feed inhibits the ability of the ICB biomass to degrade feed materials, then we can assume that effluent COD values would increase, causing an increase in toxicity. In Figure 5, the effluent COD was plotted with effluent toxicity to determine if COD trends could be related to toxicity changes. COD concentrations decreased from days 108 to 121, yet the toxicity of the effluent had various peaks and valleys. From days 129 to 136, COD remained relatively constant. However, the toxicity increased (negative peak) and then decreased (day 137). In several areas, the effluent COD increased while the toxicity decreased (days 136-144). There were no obvious trends in COD that could be related to toxicity.

Even though the ICB could maintain the proper reduction in COD, there may be trace amounts of by-products from the more toxic feed components, causing negative peaks in toxicity. In Figure 6 vertical lines were added to indicate where the changes in feed formulations occurred. Since the HRT was 5 days, the vertical lines have been shifted 5 days to show what feed formulation produced the particular effluent. The HDH, TET, and H_2O feed components were considered the base formulation and changed gradually over time, allowing for acclimation of the ICB biomass. However, the CST and DPE components had drastic changes that may not have allowed for biomass acclimation and perhaps were the cause of toxicity variability. When comparing the changes of CST and DPE in the feed formulations to changes in toxicity, trends could not be determined.
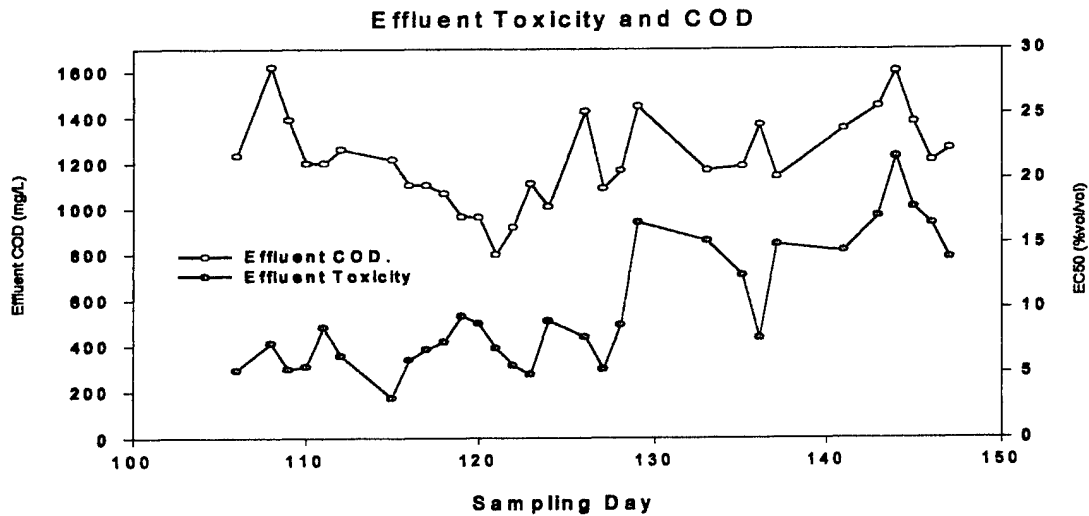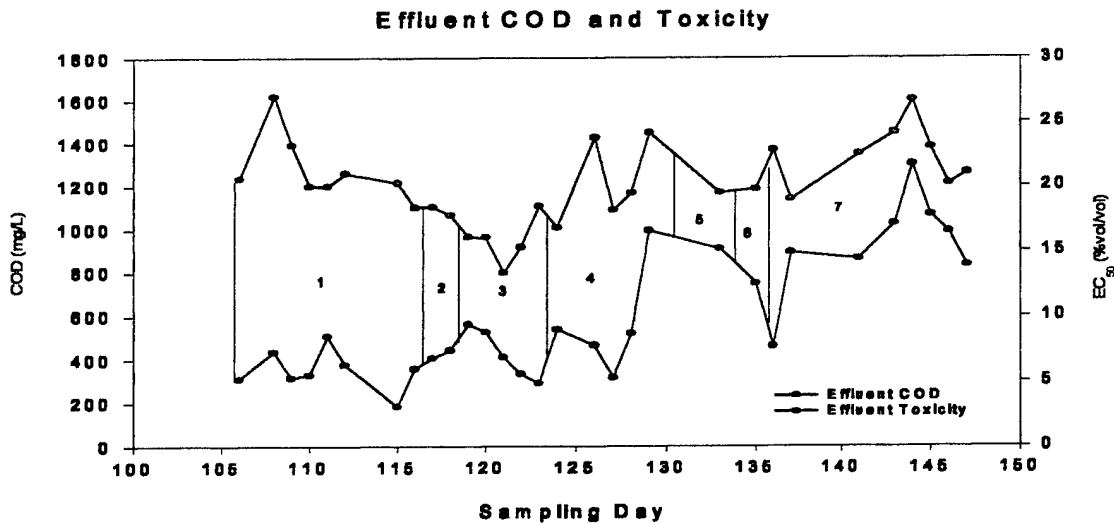
Figure 5. Effluent Toxicity and COD Plotted to Determine if Trends Exist to Cause the Negative Peaks.

#	HDH	TET	CST	DPE	H2O
1	40 gal	1.8 gal	0.5 gal	0.0 gal	134.5 gal
2	40 gal	1.8 gal	2.0 gal	0.0 gal	133.0 gal
3	40 gal	1.0 gal	2.0 gal	0.0 gal	132.0 gal
4	45 gal	1.0 gal	0.0 gal	2.0 gal	127.0 gal
5	50 gal	1.0 gal	3.0 gal	0.0 gal	121.0 gal
6	50 gal	1.0 gal	2.0 gal	1.0 gal	121.0 gal
7	50 gal	1.0 gal	0.0 gal	3.0 gal	121.0 gal

Figure 6. Effluent COD and Toxicity Plotted with Vertical Lines Depicting Feed Change. The numbers between the curves correspond to the feed type shown in the above chart.

4.   DISCUSSION

The ICB was able to reduce the toxicity of all the feed formulations throughout the study. However, the reduction in toxicity never reached a steady state. As shown in Figure 6, there were numerous positive and negative peaks in toxicity. Many appear to be oscillations around a central point; however, two increases in toxicity on days 119-123 and 129-126 cannot be explained. Since these negative trends consist of several points, it appears to be a real phenomenon. It was assumed that if the feed inhibited the ability of the ICB biomass to degrade feed materials, the effluent COD values would increase, causing an increase in toxicity. However, this may not be a valid argument for the cause and effect. The most toxic component may contribute little to the COD. If the degradation process is altered by a toxic component, the ability to degrade nontoxic materials in the feed would lead to a high COD without increased toxicity. Unsuccessful attempts were made to relate effluent COD and the change in feed components to the toxicity of the effluent.

There could be several reasons why the effluent toxicity had many peaks and valleys. Only one sample from the feed and effluent was collected at the same time each day. Perhaps sampling several times a day to generate more data points would reduce the variation in effluent toxicity. Several of the feed formulations were only used for one or two days, thus generating only one data point for a particular formulation, which greatly increased the variation between samples. There were also more than one component changed at the same time, making it impossible to relate the change in toxicity to a single component.

In Figure 6, the vertical lines depicting the change in feed composition were placed on the graph as if the formulation change was instant. In fact, each new batch of feed consisted of 175 gal of new material mixed with 25 gal of the previous material that remained at the bottom of the feed tank. The entire tank was mixed for 30 min, then pumped into the ICB over a 24-hr period. Not only is the new feed blended with the old when preparing the new batch, the process of adding the feed to the ICB also blends old with new. Therefore, when the feed formulation is changed, the division between the formulation is indiscriminant. Taking the blending of feed formulations into account, the effluent toxicity results were transformed (Figure 7) to a 5-day moving average (5 days were used due to the 5-day HRT). Using 5-day moving average data, the oscillations in the effluent toxicity curve are nonexistent, and the interpretation of data becomes much easier. The representation of the data in this manner clearly shows the increased reduction in toxicity to be more evident over time. With the addition of more toxic components to the feed, the ICB was able to maintain a steady reduction in toxicity. The continued reduction in toxicity indicates that the ICB may not have reached peak performance regarding toxicity reduction.

It appears the MTX assay is a viable tool for monitoring the toxicity reduction in effluent. However, it is not known if the MTX assay could determine if there was a system failure. During this study, there were no major system failures to cause the ICB to produce effluent with a COD removal < 90%. Future studies are needed to determine if the MTX assay can discriminate between normal operations and a failure. A feed formulation needs to be used for an extended period to create a substantial toxicity baseline. Then, force the bioreactor to fail by adding a biocide and determine the effects (toxicity and COD) on the effluent.
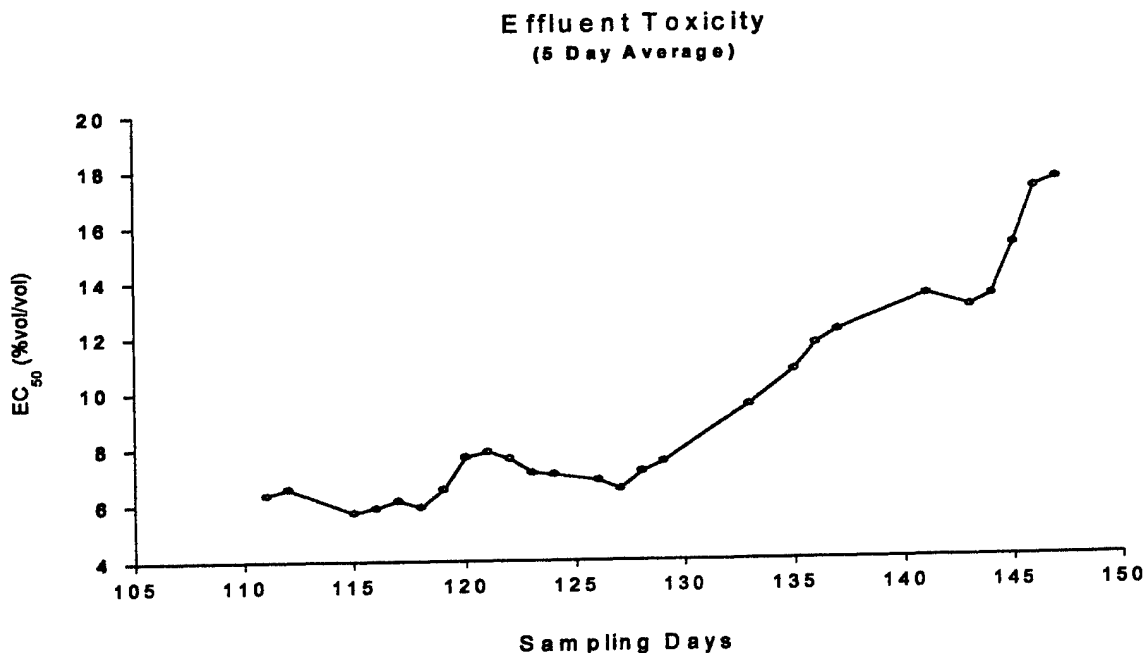
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Effluent Toxicity
(5 Day Average)

Figure 7. Effluent Toxicity Plotted as a 5-Day Running Average. This was done to account for the blending of feed batches.

5.   CONCLUSIONS

As a result of testing performed, the following conclusions are provided:

- Feed toxicity ranged from 0.6-1.2% throughout the toxicity testing, while the effluent toxicity ranged from 5.7% at the start of testing to 17.5% at the conclusion. The feed components were subjected to Microtox (MTX) assay separately in an attempt to determine which one contributed the most toxicity to the feed. The order of toxicity from highest to lowest was Continuous Stream Treatment (CST) with shredded wood pallets, Tetrytol, Hydrolyzed HD, and continuous stream treatment with designated protective equipment (DPE). The addition/change of component concentrations did not alter the ability of the Immobilized Cell Bioreactor (ICB) to reduce toxicity. It was not evident in the effluent toxicity results when the feed components were changed.

- The daily MTX effluent toxicity results produced oscillating curves that were difficult to interpret. Trends in toxicity could not be related to the chemical oxygen demand (COD) concentrations or feed component additions. The addition of new feed batches occurs through a blending of feed used in prior additions. Since the hydrolic resonance time (HRT) was 5 days, the effluent toxicity results were transformed to 5-day averages. Treating the data this way, the blending of feed batches was accounted for, and data interpretation was simplified.

16

- Water Hydrolysis of Energetic and Agent Technology (WHEAT) has demonstrated the ability of the ICB to biodegrade hydrolyzed HD and tetrytol. Neither hydrolyzed mustard nor tetrytol was ever detected in the effluent streams. Based on toxicity results, the bioreactor had not reached peak performance. The reduction in toxicity was increasing over time and did not reach a plateau at the end of testing.

- The MTX assay has proven to be a viable screening tool for monitoring the toxicity of bioreactor effluents. More refined studies are needed to determine if the MTX assay could detect failures in the biodegradation process. Consistent effluent toxicity data needs to be generated from a one-feed formulation. Once the toxicity results have stabilized, a change in the feed should be made to disrupt the biodegradation process and determine the effects on the effluent COD and toxicity.

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LITERATURE CITED

1. Harvey, S.P.; Szafraniec, L.L.; Beaudry, W.T.; Earley, J.T.; and Irvine, R.L. *Neutralization and Biodegradation of Sulfur Mustard*; ERDEC-TR-388; U.S. Army Edgewood Research, Development and Engineering Center: Aberdeen Proving Ground, MD, 1997; UNCLASSIFIED Report (AD-A322638).

2. Guelta, M.A.; Defrank, J.J. *Performance of Immobilized Cell Bioreactors for Treatment of HD and VX Hydrolysates;* ERDEC-TR-497; U. S. Army Edgewood Research, Development and Engineering Center: Aberdeen Proving Ground, MD, 1998; UNCLASSIFIED Report (AD-A349080).

3. Guelta, M.A.; Chester, N.A.; Lupton, S.; Koch, M.; and Fry, I.L. *Biodegration of HD and Tetrytol Hydrolysates in a Pilot Scale Immobilized Cell Bioreactor*; ECBC-TR-192; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2001; UNCLASSIFIED Report (AD-A396602).

4. Haley, M.V.; Kurnas, C.W.; Ware, J.A., Sr. *Toxicity of a Biodegraded Chemical Warfare Agent (Mustard) to Aquatic Organisms;* ECBC-TR-002; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2000; UNCLASSIFIED Report (AD-A381086).

5. Haley, M.V.; Kurnas, C.W.; Turley, S.D.; Burton, D.T. *Federally Owned Treatment Works (FOTW) Demonstration Test, Chronic Aquatic Toxicity of HD Bioeffluents*; ERDEC-TR-553; U.S. Army Edgewood Research, Development and Engineering Center: Aberdeen Proving Ground, MD, 1998; UNCLASSIFIED Report (AD-A361552).